Phytochemical investigation of Impatiens *rothi* and Impatiens *tinctoria* crude extracts and evaluation of their antibacterial activities

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ABSTRACT

Background: Medicinal plants remain a critical source of therapeutic agents with their applications supported by indigenous knowledge and ethnobotanical evidence. However, there is limited evidence on the phytochemical profile of impatiens *rothi* and impatiens *tinctoria* root extracts. Thus, this study evaluated the phytochemical profile and in vitro antibacterial activity of impatiens *rothi* and impatiens *tinctoria* root extracts.

Methods: The roots were collected from Kuyu District, North Shewa Zone, Oromia Regional State, Ethiopia. The plants were dried, powdered, and extracted using chloroform, ethyl acetate, hexane, methanol, ethanol, and distilled water. Standard phytochemical assays were conducted to detect major classes of secondary metabolites, while antibacterial activity was assessed against gram-positive and gram-negative bacteria using the disc diffusion method. Minimum inhibitory concentration (MIC) values were determined by broth microdilution. Screening revealed alkaloids, flavonoids, tannins, phenolics, saponins, and terpenoids in most extracts, with ethanol and methanol extracts presenting the richest profiles. The data was analyzed using mean and standard deviation and analysis of variance (ANOVA).

Results: Ethanol extract of impatiens *tinctoria* demonstrated the highest mean antibacterial activity (19±0.35 mm) against *Pseudomonas aeruginosa*, comparable to chloramphenicol (26 mm) and vancomycin (20 mm). Conversely, the lowest mean inhibition zone (7.2±0.28 mm) occurred with hexane extract of impatiens *rothi* against *Pseudomonas aeruginosa*. The lowest MIC (3.50mg/mL) was recorded in chloroform extract of impatiens *rothi* against *Escherichia coli* and in aqueous extract of impatiens *tinctoria* against *Pseudomonas aeruginosa*. Antibacterial effects varied significantly across solvents, highlighting the influence of the polarity on extracting bioactive compounds.

Concussion: Impatiens roots contain diverse phytochemicals with promising antibacterial potential, justifying their ethnomedicinal use. Ethanol and chloroform extracts were most effective against *Pseudomonas aeruginosa* and *Escherichia coli*. Further phytochemical characterization and toxicity assessments are recommended to validate active constituents and ensure therapeutic safety.

Keywords: Traditional drugs, Antibacterial, Impatiens *rothi*, Impatiens *tinctorial*, Phytochemicals

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BACKGROUND

Medicinal plants are globally recognized as important sources of antimicrobial agents, especially in regions where access to conventional medicines is limited (1). Traditional medicine plays a vital role in both human and animal health care in Ethiopia, where cultural acceptability, economic constraints, and local availability make plant-based medicines a first choice for many communities (2). A recent systematic review in Ethiopia found that about 65% of the Ethiopian population use traditional medicine, and among these, some 95% of the preparations are plant-based (3). This underscores that plant-based remedies remain crucial for primary health care. Moreover, another systematic review highlighted the broad antibacterial and antifungal potential of Ethiopian medicinal plants, further emphasizing their importance in managing infectious diseases in the country (4).

Alongside the reliance on traditional remedies, there is a growing global concern about the emergence of antimicrobial resistance (5). Many bacteria that were once easily treatable are now developing resistance to multiple antibiotics, both in Gram-positive and Gram-negative groups (6). This problem is compounded in developing countries by the limited access to newer or more expensive drugs, making the need for alternative antimicrobial sources more urgent. Research has shown that plant secondary metabolites such as alkaloids, terpenoids, flavonoids, tannins, phenolics, and saponins possess antimicrobial activity, but their effectiveness depends greatly on plant species, plant part, solvent used for extraction, and the microbial strain tested (4, 6).

In Ethiopia, *Impatiens tinctoria (I. tinctoria*), locally known by the name "Ensosila", is among the medicinal plants traditionally used for skin infections, fungal conditions, and as a dye for cosmetic purposes (nail, nail beds, hands). A study evaluated the antibacterial and acute oral toxicity of root extracts of *I. tinctoria*, using aqueous, ethanol, and ethyl acetate solvents and found that Gram-positive bacteria were more susceptible than Gram-negative with the ethyl acetate extract showing the strongest activity among the extracts tested (7). The extracts were practically non-toxic at high doses (LD_{50} above 9600 mg/kg in mice) (7). Similarly, another study demonstrated that the tuber of *I. tinctoria* has significant antioxidant and mineral properties, and reported its antifungal and antibacterial potential in preliminary assays. highlighting bioprospecting potential and therapeutic uses in Ethiopia (8, 9).

Despite these promising results for *I. tinctoria*, less is known about *Impatiens rothi*, especially its root extracts, their phytochemical properties, and their comparative antibacterial effectiveness. Traditional healers living in the study area are using the roots of both *I. rothi* and *I. tinctoria* to treat bacterial infections, yet there is limited published data on their relative performance across different solvents, or against both Gram-negative and Gram-positive pathogens. However, recent studies have begun evaluating *I. rothi*, demonstrating its analgesic, anti-inflammatory, and antimicrobial potential (10).

Moreover, the choice of solvent in extracting plant materials can dramatically affect yield and diversity of bioactive compounds (11). Polar solvents such as ethanol and methanol tend to extract phenolics, flavonoids and other polar compounds more efficiently, whereas non-polar solvents like hexane and chloroform may yield lipophilic compounds (terpenoids, some alkaloids). Testing multiple solvents across a range of polarity allows more comprehensive phytochemical screening and may uncover compounds with differing spectra of antibacterial activity (6). Thus, with antimicrobial resistance rising, there is a pressing need to discover and validate new antimicrobial agents from traditional sources such as medicinal plants used in Ethiopia. However, several traditional medicines including I. rothi have not been fully evaluated.

Therefore, this study aimed to conduct a comprehensive phytochemical screening of root crude extracts from *I. rothi* and *I. tinctoria* to identify major secondary metabolites and evaluate the efficiency of various solvents in extracting bioactive compounds. Furthermore, it investigated *in vitro* antibacterial activity of these extracts, against four pathogenic bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*,

Staphylococcus aureus, and *Streptococcus pneumoniae.*

MATERIALS AND METHODS

Study area

This study was conducted in the Microbiology Laboratory of Salale University. Plant samples were collected from Kuyu District, North Shewa Zone, Oromia Regional State of Ethiopia. The geographical coordinates of the district are 9°52′0″ N latitude and 38°21′0″ E longitude. The district covers an area of 6981.8 km^2. The district experiences a mean annual temperature of 19.5 °C and comprises 19 rural villages and one administrative town (Garba Guracha Town).

Plant material collection and preparation

Roots of *I. tinctoria* and *I. rothi* were selected based on ethnomedicinal knowledge, as traditional healers in the study area frequently recommend these plants for antibacterial purposes. Four kg of fresh roots of each species were collected, cleaned thoroughly to remove soil and debris, and cut into small pieces. After drying at room temperature for one month, 1kg of root material from each plant was obtained for further analysis (Fig 1).

Fiure 1a: Dried and chopped root of *I. rothi*:
Figure 1b: Dried and chopped root of *I. tinctoria*

Extraction procedure

The dried roots were ground into fine powder using an electric grinder and sieved through a 30 µm mesh. For extraction, 50 g of powdered root material was mixed with 150 mL of each solvent (distilled water, ethanol, methanol, chloroform, hexane, and ethyl acetate) in a 1:3 ratio. The mixtures were subjected to continuous agitation on an orbital shaker for 8hr daily for 3 consecutive days. Extracts were filtered using Whatman No. 1 filter paper under vacuum, and filtrates were concentrated by rotary evaporation followed by oven drying at 40 °C. Dried extracts were weighed and reconstituted in 50% dimethyl sulfoxide (DMSO) to obtain a stock concentration of 100 mg/mL, which was stored at 4 °C until use.

Chloroform extracts were dissolved by microwave-assisted vertexing to ensure complete solubilization (Fig 2).

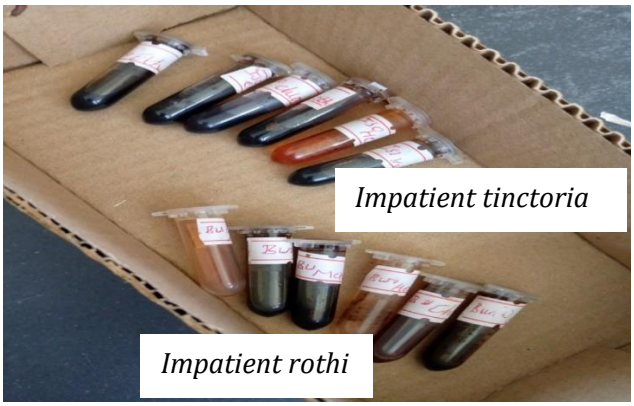

Figure-2: solvent extracted and dissolved in 50% of DMSO of the plant crude extract

Phytochemical screening

Qualitative phytochemical screening was performed to detect major bioactive compounds following standard procedures (12). Dragendorff's test was performed by adding Dragendorff's reagent to the extract, where the formation of an orange or yellow precipitate confirmed the presence of alkaloids (13). For saponins, extracts diluted to 20 mL with distilled water were vigorously shaken in a graduated cylinder for 15 minutes, and the formation of a persistent 2 cm foam layer indicated their presence (14). Tannins were detected by boiling 0.5 g of extract in 20 mL of distilled water, filtering, and treating the filtrate with 0.1% ferric chloride solution; a blue-black or greenish coloration confirmed tannins (15). Flavonoids were identified when extracts treated with dilute NaOH developed a yellow color that disappeared upon acid addition, and precipitation with lead acetate further confirmed their presence (16). Phenolic compounds were also confirmed when extracts treated with 10% ammonium hydroxide produced a yellow fluorescence (16).

Microorganisms

Antibacterial activity was assessed against two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumoniae* (ATCC 63), and two Gram-negative bacteria: *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (clinical isolate). Strains were obtained from the Ethiopian Public Health Institute (EPHI) and maintained on nutrient agar slants at 4 °C until use (17).

Culture media preparation and inoculum standardization

According to instructions of the manufacturer, Mueller–Hinton Agar (MHA) was prepared by dissolving 38 g of MHA powder in 1 L of distilled water, adjusting the pH to 6.8, and sterilizing at 121 °C for 15 min. After cooling to 50 °C, 25 mL of medium was poured aseptically into sterile 90 mm Petri dishes. Randomly selected plates were incubated at 37 °C for 24 hrs to confirm sterility (18). Sets of activities were conducted during media preparation (Fig 3).

Pure colonies of each test bacterium were inoculated into nutrient broth and incubated at 37 °C for 2 hrs. The turbidity was adjusted to 0.5 McFarland standard (≈1.5 × 10^8 CFU/mL) using sterile broth and verified spectrophotometrically at 625 nm (17).

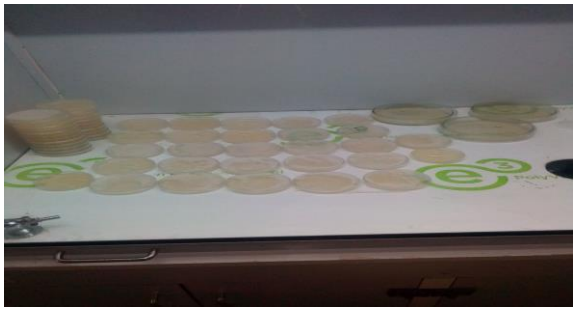

Figure-3: Media preparation

Preparation of discs and antibacterial susceptibility test

Sterile 6 mm discs (Whatman No. 1 filter paper) were impregnated with 30 µL of plant extract (100 mg/mL), air-dried, and stored at 4 °C in sterile containers. Chloramphenicol (30 µg/disc) and vancomycin (30 µg/disc) served as positive controls, while solvent-only discs served as negative controls (17).

The antibacterial activity of extracts was evaluated using the Kirby–Bauer disc diffusion method (Bauer *et al.,* 1996). Standardized inoculate were swabbed uniformly onto MHA plates, and discs containing extracts and controls were aseptically placed on the inoculated surface. Plates were incubated at 37 °C for 24 hours, and the diameter of inhibition zones (including disc diameter) was measured in millimeters using a ruler.

Minimum inhibitory and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) values were determined using the broth microdilution method (18, 19). Serial twofold dilutions of plant extracts (100–1.56 mg/mL) were prepared in Mueller Hinton Agar. Each tube was inoculated with 20 µL of standardized bacterial suspension and incubated at 37 °C for 24 hr. The lowest concentration showing no visible growth was recorded as the MIC.

Aliquots from MIC tubes with no visible growth were subculture onto MHA plates and incubated at 37 °C for 24 hr. The lowest extract concentration yielding no bacterial growth was considered the minimum bactericidal concentration (MBC).

Statistical analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Duncan's multiple range test was performed using SPSS version 20. Mean differences were considered statistically significant at $p < 0.05$.

RESULTS

Preliminary phytochemical screening

Phytochemical analysis of *I. tinctoria* and *I. rothi* revealed the presence of major secondary metabolites with variation depending on solvent polarity. In *I. tinctoria*, flavonoids, phenols, tannins, alkaloids, and saponins were consistently detected, whereas *I. rothi* showed the presence of flavonoids, phenols, tannins, and alkaloids, but saponins were absent. The detailed results of phytochemical screening are presented in (Table 1 and Table 2).

Table-1: Preliminary phytochemical screening of *Impatiens tinctoria* crude extracts
(+ indicates presence; – absence)

Phytochemical	***Ethanol***	***Methanol***	***Chloroform***	***Ethyl acetate***	***Hexane***	***Water***
Flavonoids	+	–	+	+	+	–
Tannins	+	+	–	–	+	+
Alkaloids	+	+	+	+	–	+
Saponins	–	+	–	–	–	–
Phenols	+	+	–	+	+	+

Table-2: Preliminary phytochemical screening of *Impatiens rothi* crude extracts (+ indicates presence; – absence)

Phytochemical	***Ethanol***	***Methanol***	***Chloroform***	***Ethyl acetate***	***Hexane***	***Water***
Flavonoids	+	–	+	+	+	+
Tannins	–	+	+	–	–	+
Alkaloids	+	+	+	–	–	+
Saponins	–	–	–	–	–	–
Phenols	+	+	–	+	+	–

Antibacterial activity assay

The antibacterial activities of six solvent extracts (water, ethanol, methanol, chloroform, hexane, and ethyl acetate) at 50 mg/mL concentration were evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*. Table 3 and Table 4 depict the antibacterial activity of the extracts. Standard antibiotics chloramphenicol (30 µg/disc) and vancomycin (30 µg/disc) served as positive controls.

In *I. rothi* hexane extracts produced the highest inhibition (14 ± 0.28 mm) against *E. coli*, followed by methanol (12 ± 0.43 mm) against *S. aureus*, chloroform (11 ± 0.62 mm) against *P. aeruginosa*, and ethyl acetate (13 ± 0.57 mm) against *S. pneumoniae*. The lowest inhibition zone (7 ± 0.53 mm) was recorded with chloroform against *E. coli* (Table 3).

Table-3: Mean inhibition zones (mm ± S.D.) of *Impatiens rothi* crude extracts

Test organism	Water	Ethanol	Methanol	Chloroform	Ethyl Acetate	Hexane	*C30*	*V30*
E. coli	–	–	–	7 ± 0.53	8 ± 0.34	14 ± 0.28	20	24
S. aureus	–	7.8±0.42	12±0.43	10 ± 0.52	9 ± 0.28	8 ± 0.36	18	16
S. pneumoniae	8±0.22	7.5±0.38	8±0.56	13 ± 0.57	9 ± 0.32	8.4±0.62	20	18
P. aeruginosa	8±0.38	7.4±0.25	9 ± 0.60	11 ± 0.62	8.7±0.30	7.2±0.28	17	20

In *I. tinctoria*, methanol extracts showed maximum inhibition against *P. aeruginosa* (19 ± 0.35 mm), chloroform against *E. coli* (15 ± 0.37 mm) and *S. aureus* (11 ± 0.38 mm). The hexane extract demonstrated the greatest inhibition against *S. pneumoniae* (16 ± 2.8 mm). In contrast the ethyl acetate extract did not show activity against *E. coli*, and the water extract was inactive against *S. aureus* **(**Table 4).

Table-4: Mean inhibition zones (mm ± S.D.) of Impatiens *tinctoria* crude extracts

Test organism	Water	Ethanol	Methanol	Chloroform	Hexane	Ethyl acetate	C30	V30
E. coli	8.3±2.8	10.3±0.83	13.8±0.56	15±0.37	12±0.46	–	25	28
P. aeruginosa	7.3±2.8	19±0.35	12.3±0.24	15.7±1.5	16.2±0.56	12±0.0	26	20
S. aureus	–	9±0.04	10±0.46	11±0.38	10±0.38	9±0.0	20	19
S. pneumoniae	14±2.8	12±1.5	11±0.16	15±0.0	16±2.8	12±0.58	24	21

Minimum inhibitory and minimum bactericidal concentration

The MIC and MBC values of the crude extracts further confirmed their antibacterial potential (Table 5). Both *I. rothi* and *I. tinctoria* showed MIC and MBC values ranging between 3.5–3.9 mg/mL for all test organisms (Fig 6).

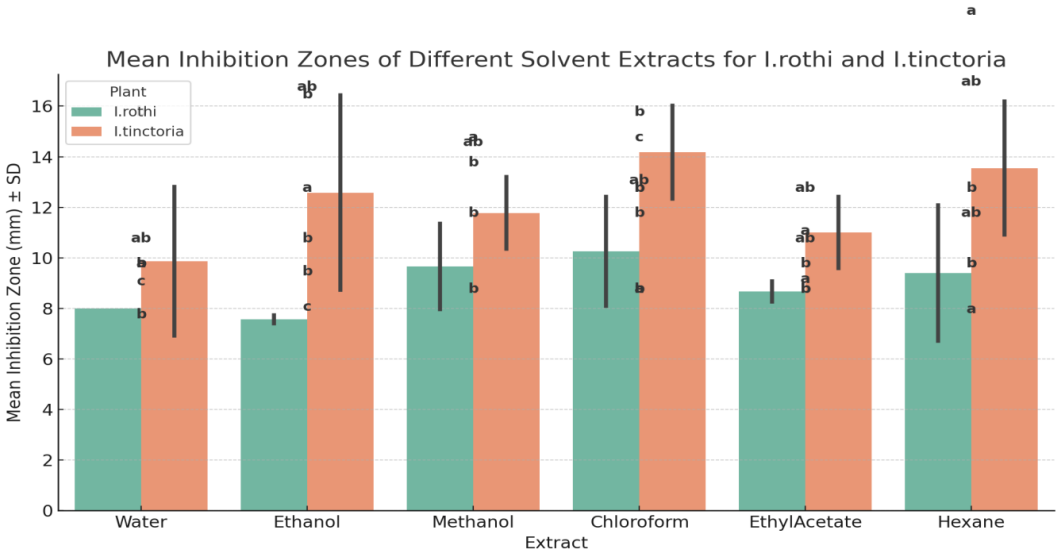

Figure-6: Inhibition zones of the plants on selected bacteria

Table-5: MIC and minimum bactericidal concentration values (mg/mL) of *Impatiens rothi* and *Impatiens tinctoria* crude extracts

Test organism	Gram type	*Impatiens rothi* MIC	*Impatiens rothi* MBC	*Impatiens tinctoria* MIC	*Impatiens tinctoria* MBC
E. coli	–	–	3.50	3.50	3.50
S. aureus	+	3.75	3.75	3.90	3.90
S. pneumoniae	+	3.75	3.75	3.75	3.75
P. aeruginosa	–	3.60	3.60	3.60	3.60

MBC-minimum bactericidal concentration; MIC-Minimum inhibitory concentration

Table-6: combined two-way ANOVA (Plant × Organism × Extract) of inhibition zone data

Source of Variation	**df**	**Sum of Squares**	**F**	**p-value**
Plant (*I. rothi* vs *I. tinctoria*)	1	112.90	**17.36**	**0.0019**
Organism (*E. coli, S. aureus, S. pneumoniae* and *P. aeruginosa*.)	3	34.02	1.74	0.2241
Extract solvents	5	69.80	2.15	0.1493
Plant × Organism	3	40.28	2.06	0.1688
Plant × Extract	5	24.77	0.76	0.5974
Organism × Extract	15	70.44	0.72	0.7195
Residual	10	65.05		

The type of plant had a highly significant effect on antibacterial activity ($p = 0.0019$). This indicates that the inhibition zones produced by *I. rothi* and *I. tinctoria* differed significantly. In contrast, the type of bacterial organism did not have a significant effect ($p = 0.2241$). The activities of the extracts were relatively consistent across *E. coli*, *S. aureus*, *S. pneumoniae*, and *P. aeruginosa*. The type of extract solvent also showed a non-significant effect ($p = 0.1493$). This indicates there were also some differences among solvents, these were not statistically significant at the $p < 0.05$ level. In addition, none of the interactions between factors plant × organism, plant × extract, or organism × extract. The patterns of antibacterial activity remained consistent across all combinations of plants, organisms, and extracts.

DISCUSSION

Ethnobotanical screening continues to be a productive strategy for identifying new antibacterial leads from plants used in traditional practice. The present study evaluated the in vitro antibacterial activity of *I.rothi* and *I.tinctoria*. These two species used locally as cleansing/detergent plants. Crude extracts produced inhibition zones of 7–19 mm and MIC/MBC estimates in the order of 3.5–3.9 $mg \cdot mL^{-1}$, with *I. tinctoria* consistently showing stronger activity than *I. rothi*, particularly for methanolic and ethanolic extracts. However, aqueous extracts were the least active.

Several complementary explanations are consistent with these findings. For instance, the extraction solvent strongly influences the activity of the extract-activity by determining which classes of secondary metabolites are recovered (14, 20): Polar organic solvents such as methanol and ethanol typically solubilize phenolics, flavonoids, tannins and glycosides more efficiently than water, and often produce extracts with higher antimicrobial potency (21, 22). Bacterial envelope architecture modulates susceptibility could also affect the activity of plant extracts. For example, Gram-negative organisms possess an outer membrane containing lipopolysaccharide that functions as a permeability barrier, often rendering them less sensitive to hydrophobic or larger plant molecules, whereas Gram-positive bacteria lack this barrier and therefore tend to show larger zones of inhibition to many plant extracts (14). In agreement with this pattern, our results show generally larger inhibition zones against Gram-positives while

retaining measurable activity against Gram-negative strains, implying that some components in the impatiens extracts are capable of permeating or otherwise circumventing Gram-negative defenses.

Our observations accord with prior work on impatiens and related genera. It was also reported significant antibacterial activity for I. tinctoria root fractions (notably ethyl acetate), with methanolic and ethanolic extracts also active and with favourable acute-toxicity profiles in mice (7). Similarly, polyphenolic and flavonoid-rich extracts from several Impatiens species have been linked to antibacterial and antioxidant activities (23). The co-occurrence of antioxidant and antibacterial activity is mechanistically credible because phenolics can disrupt microbial membranes, chelate metal ions required for microbial metabolism, and interfere with essential enzymes, while antioxidant capacity often correlates with the overall redox-active phytochemical load (21, 24).

Flavonoids, tannins and phenolic acids detected in many Impatiens extracts have well-documented antibacterial mechanisms including membrane perturbation, inhibition of nucleic acid synthesis, and enzyme inhibition; reviews emphasize both direct bactericidal activity and synergistic potentiation with existing antibiotics (25). The comparatively superior activity of *I. tinctoria* in our study, therefore, likely reflects either higher concentrations of these bioactive phenolics and flavonoids, or the presence of particular active molecules (for example, naphthoquinones and related compounds reported in some impatiens species) with favorable permeability and bactericidal effects (23).

Although crude-extract MICs in our study (3.5–3.9 $mg \cdot mL^{-1}$) are higher than typical clinical antibiotics, they are in the same magnitude as other validated plant extracts and thus justify fractionation and bioassay-guided isolation (26, 27). Fractionation often concentrates active constituents and can lower MICs to pharmacologically relevant ranges. It also allows structural identification (HPLC, GC-MS, NMR) and subsequent evaluation of mechanism of action and toxicity. Prior work with I. tinctoria identified ethyl-acetate fractions as particularly active and non-toxic at doses used in acute testing (7).

From a translational and public-health perspective, these results are meaningful. In low-resource settings where commercial antiseptics and detergents are scarce, validated local botanical detergents that exert antimicrobial activity could reduce

hygiene-related infections and promote community health, provided safety is established (5, 28). More broadly, the continuing rise of antimicrobial resistance underlines the urgency of expanding the natural product pipeline: underexplored indigenous species such as *I. tinctoria* and *I. rothi* may harbor novel scaffolds or antibiotic potentiators useful for drug development or as adjuvants (29).

The current study has some limitations. In vitro zones and MIC/MBC values do not predict in vivo efficacy, and crude extracts can contain multiple compounds that interact antagonistically or synergistically. Therefore, bioassay-guided fractionation, toxicological profiling (sub-acute and chronic), and pharmacokinetic studies are required before clinical considerations. Moreover, the activity of the extracts may vary with plant part, harvest time, drying method and geographic provenance. Thus, standardization of the extracts with variable conditions is necessary (30).

CONCLUSION

This study demonstrated that crude extracts of *I.tinctoria* and *I. rothi*, obtained using six solvents (methanol, ethanol, water, ethyl acetate, hexane, and chloroform) showed a significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Among the tested extracts, *I. tinctoria* generally exhibited stronger activity than *I. rothi*, with methanolic and ethanolic extracts showing the most consistent inhibition. The results further revealed that solvent polarity plays a crucial role in e extraction of bioactive compounds, with ethanol and methanol providing higher antibacterial activity compared to water. The inhibition zones obtained were comparable to those of standard antibiotics, supporting the traditional use of these plants as detergents and antimicrobial agents in rural Ethiopia. These findings provide scientific evidence for the potential application of *Impatiens* species in developing new, plant-based antibacterial formulations for both human and veterinary medicine. Future studies should focus on isolating and characterizing the active compounds, optimizing extraction conditions, and evaluating the antibacterial efficacy against a wider range of pathogenic bacteria. Moreover, in vivo safety and effectiveness assessments are recommended to support potential applications in pharmaceuticals, food preservation, and personal hygiene products.

List of abbreviations

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Authors' Contributions: AM conducted experiments and collected plant materials. DG conceptualized the study, conducted experiments, analysed data, and wrote and edited the manuscript. ZK, TS and SB supervised the work, provided critical comments.

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Availability of data and materials: The datasets and materials supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests: The authors declare no competing interests.

Ethics approval and plant guidelines: This study was conducted in accordance with ethical guidelines. No human or animal subjects were involved. The use of plants in this study complies with international, national, and institutional guidelines.

Permissions to collect the plants: All plant seed specimens were collected following formal requests and obtained with the necessary permissions from the relevant authorities. The names and sources of all plant seeds are specified in the Methods section.

Consent for publication: All authors have provided consent for the publication of this manuscript.

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